Expression and Function of the *ultraspiracle* (*usp*) Gene during Development of *Drosophila melanogaster*

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Abstract:

The *usp* locus encodes a member of the nuclear hormone receptor superfamily in *Drosophila melaaogaater* that interacts with EcR (ecdysone receptor) to mediate ecdysteroid-induced gene expression. A 2.7-kb usp mRNA was detected at all developmental times tested, although its abundance varied. Among premetatnorphic stages, both the 2.7-kb transcript and Usp protein attained their highest levels in the late third larval instar. The 2.7-kb *usp* transcript was also found in adult stages and a 1.2-kb transcript was detected in the polyadenylated RNA fraction of both mature adult females and early embryos. Aneuploids carrying two *asp* mutant alleles and a putative variegating usp^{+} allele often developed deformities of the adult wing disc that apparently resulted from mutational disruption of *uap* activity before metamorphosis and whose frequency was affected by maternal genotype. Both of the recessive lethal *uap* mutations associated with this "cleft thorax" phenotype involved substitutions of conserved arginine residues in the DNA-binding domain, although the frequency of the phenotype was not the same for the two alleles. Both mutant proteins retained the ability to form heterodimers with EcR *in vitro* but showed reduced affinity for an ecdysone response element.

Abbreviations used: BR-C, Broad-Complex; DBD, DNA-binding domain; EcR, ecdysone receptor; EcRE, ecdysone response element; EMS, ethylmethane sulfonate; hsp27, 27-kDa heat shock protein; PCR, polymerase chain reaction; *rp49*, ribosomal protein 49; RXR, retinoid X receptor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; tap, ultraspiracle; Usp, Ultraspiracle gene product.

Article:

INTRODUCTION

Postembryonic development in holometabolous insects requires periodic surges of the insect steroid hormone, 20-hydroxyeedysone, that stimulate cellular changes associated with molting and metamorphosis. The temporal and spatial patterns of chromosomal activity elicited by 20-hydroxyealysone in the salivary gland of *Drosophila melanogaster* and other insects reveals a highly coordinated series of transcriptional changes (Ashburner *et at,* 1974) that have been characterized molecularly (Huet *et at,* 1993). Nevertheless, the heterogeneity of ecdysteroid-dependent transcription at both the tissue and organismal levels involve regulatory events that remain unexplained (Karim and Thummel, 1992; Andres *et al,* 1993).

The action of 20-hydroxyeedysone depends upon its interaction with a heteromeric complex composed of two members of the nuclear hormone receptor superfamily, EcR3 (Koelle *et al,* 1991) and Usp, the product of the *usp* gene locus (Yao *et al,* 1993). This complex recognizes EcREs *in vitro* (Yao *et al,* 1992; Thomas *et al.,* 1993; Tal- bot *et* al, 1993) and puff sites associated with early ecdy- steroid-induced puffs in vivo (Yao et *al.,* 1993). lisp is a homologue of the mammalian RXRs, which themselves can form heteromers with several vertebrate nuclear re- ceptors to modulate their affinity for genomie response elements (Yu *et al,* 1991; Kliewer *et* at, 1992; Bugge *et* al, 1992; Leid *et* al, 1992; Marks *et at,* 1992; Zhang *et al,* 1992; Yao *et at,* 1992). Among the transcriptional regu- lators induced by 20-hydroxyecdysone, some are other members of the receptor

superfamily (Segraves and Hogness, 1990; Lavorgna et a, 1993; Stone and Thum- mel, 1993), raising the possibility that the ecdysone receptor complex undergoes later modifications that alter its activity (Richards, 1992). Like Usp, these are orphan receptors for which no known hormone ligand has been identified (see Segraves, 1991). However, ectopic expression of usp^{+} during *Drosophila* embryogenesis evokes no mutant effects, implying that its function also requires the presence of a localized ligand (Oro *et al.*, 1992).

Genetically, the *usp* locus is defined by three recessive lethal mutations that cause death at the end of the first larval instar, Mutant larvae often fail to shed the first instar cuticle which then appears as an extra row of posterior spiracles (Perrimon *et al.,* 1985). In fact, the completion of ernbryogenesis requires *usp* activity supplied through the maternal germ-line (Perrimon *et al.*, 1985) and Usp has been implicated in the regulation of s15 chorion gene expression (Shea *et al.,* 1990; Khoury Christianson *et al.,* 1992). The *usp* gene plays a vital role in early stages of postzygotic development since adult gynandromorphs with mutant *usp* imaginal clones cannot be recovered (Oro *et al,* 1992). Although normal *usp* function is also required for metamorphosis, *usp* mutant thoracic clones generated during larval development are normal in both appearance and frequency (Oro *et al.,* 1992). Therefore, it is conceivable that *usp* activity is not required for all ecdysteroid-induced metamorphic events. Mutant eye-antennal clones are also recovered, but these are abnormal as the consequence of a nonautonomous effect (Oro *et al.,* 1992).

The central role that Usp plays in the regulation of hormonal processes necessitates a detailed analysis of *usp* expression and the functional effects of *usp* mutations. These studies describe the dynamics of *usp* transcription and Usp translation, reveal indications of transcriptional complexity in adult females and early embryos, identify a maternal and early embryonic critical period during which normal *usp* function is required for eventual adult thoracic development, and establish the molecular and functional basis of two lethal us]) mutations that do not exert equivalent effects on thoracic development.

MATERIALS AND METHODS

Stock preparation and timing of larvae. Several hundred *D. melanogaster* adults (Canton-S strain) were reared at 25°C for 4-5 days following eclosion and then transferred to 150-mm petri plates containing an agar- grape medium (37.5 ml concentrated grape juice, 112.5 ml water, 6 g agar) at 25°C. Females were allowed to lay eggs for approximately 1 hr. These eggs were discarded and a second 3-hour egg collection was made. Two more egg collections followed, yielding samples of early embryos (0-3 hr), mid embryos (8-11 hr), and late embryos (19-22 hr) that were frozen in liquid nitrogen and stored at —80°C until the time of RNA extraction.

For larval stagings, early first instar larvae were collected as they hatched from agar plates during a 2-hour interval (22-24 hr after egg laying). These larvae were transferred to standard cornmeal media, allowed to develop for the appropriate time at 25°C, and then separated by washing the food through sieves that retained the larvae. The mouthhooks and anterior spiracles of 20 individual larvae recovered at each time were observed in order to verify the developmental stage (Roberts, 1986). Collections were discarded if any individual in the sample was not at the appropriate stage. Otherwise, the collected larvae were frozen in liquid nitrogen. Early wandering stage larvae were chosen individually as they wandered off the food over a 6-hour period and periodically frozen in batches that were later combined. Similarly, individual late wandering larvae showing more sluggish wandering activity were collected and frozen later, as were white prepupae undergoing anterior spiracle eversion. Other white prepupae were allowed to develop for another 21-24 hr (early pupae) or 63-68 hr (late pupae) before collection. Using these methods, collections were made for early first larval instars (25-27 hr after egg laying), late first instars (39-43 hr), early second instars (46-49 hr), late second instars(60-63 hr), early third instars (72-80 hr), mid third instars (94 hr), early wandering third instars (106-112 hr), late wandering third instars (110-116 hr), white prepupae (118 hr), early pupae (139-142 hr), and late pupae (181-186 hr). Adult flies were harvested by clearing containers and allowing adults to eclose for 4-6 hr. Sexes were separated, frozen in liquid nitrogen, and stored at —80°C until the time of RNA extraction, Other newly eclosed males and females were separated and kept in vials for 4 days before freezing them.

RNA isolation. Total RNA was extracted using the hot phenol/chloroform method described previously (Jewett, 1986). Polyadenylated RNA fractions were separated using the polyATtract mRNA Isolati0n System (Promega). The manufacturer's protocol was followed at room temperature, which results in the retention of mRNA species with polyadenine tails exceeding approximately 25 nucleotides. The nonpolyadenylated fraction from these isolations was also retained for further analysis. The yield of polyadenylated RNA among samples ranged between 0.28 and 0.76% of the total RNA fraction.

Northern blotting. For Northern blots, 15 μg of total RNA, 15 μg of nonpolyadenylated RNA, or 2 μg of polyadenylated RNA from each developmental stage was loaded onto a 1.0% denaturing agarose gel containing 1X gel running buffer (0.5 *M* formaldehyde, 20 m*M* Mops, 8 m*M* sodium acetate, pH 5.0, 1 m*M* EDTA). The samples were denatured prior to loading by incubating them for 15 min in 0.5X Mops buffer containing 2.2 *M* formaldehyde and 50% formamide at 65°C. After electrophoretic separati0n, the gel was denatured (150 m*M* NaCl, 50 m*M* NaOH) and then neutralized (150 m*M* NaCl, 100 m*M* Tris, pH 8.0) and transferred with 20x SSC to Hybond- N nylon membranes (Amersham) by capillary elution. Nucleic acids were membrane-bound with 254 nm ultraviolet light in a Stratalinker 1800 (Stratagene).

After transfer, membranes were hyhridized under high stringency conditions (50% formamide, 5× SSPE, 5× Denhardt's, 1% SDS, 100 μg/ml of denatured, sheared salmon sperm DNA at 42°C) with DNA probes prepared by random priming (U.S. Biochemical). For the *usp* probe, a 2.2-kb *Eco*RI fragment was isolated by gel purification from a cDNA clone containing the entire *usp* coding region as well as flanking untranslated regions (Henrich *et al.,* 1990). The probe for a ribosomal protein *(rp49)* was prepared from a 639-bp *Eco*RI/ *Hind*III fragment that includes an entire cDNA (O'Connell and Rosbash, 1984) and used as a control for the amount of RNA which had been loaded in each lane.

After hybridization, filters were successively washed at 42°C in (i) 5× SSPE, (ii) 1× SSPE, 0.5% SDS and (iii) 0.1X SSPE, 0.5% SDS. The hybridized DNA was then visualized by exposure of the membrane to autoradi0graphic film (Kodak X-OMAT) at –80°C with intensifying screens.

A probe prepared from the common exon region of the EcR gene (Andres *et al.,* 1993) was tested with the Northern blots in order to verify the staging of the samples. The observed developmental pattern was consistent with previous reports *except* that a peak of EcR transcript occurred in the early second larval instar on these blots, whereas this peak is associated with late second instar larvae in other stagings (data not shown; Koelle *et al,* 1991; Talbot *et al.,* 1993).

Western blot analysis. Some of the staged and frozen animals described earlier were washed with 0.9% NaCl and 0.1% Triton X-100. After homogenizing in cracking buffer (0.125 *M* Tris-HC1, pH 6.8, 5% mercaptoethanol, 0.1% Triton X-100, 4 *M* urea, 1 m*M* PMSF), supernatants were collected by centrifuging at 14,000*g* for 30 min. After the protein contents were determined (Bio-Rad Protein Assay kit), SDS was added to a final concentration of 2%. After incubation for 5 min at 95°C, 80 mg of protein was applied to each slot on a 10% SDS-polyacrylamide gel. Following SDS-PAGE, protein was blotted to a ProtBlott membrane (Applied Biosystems). Nonspecific binding was blocked by pretreating the membrane in 5% nonfat dry milk in TBS (25 mM Tris-HC1, 0.5 *M* NaCl, pH 7.5) for 1 hr. Blots were then incubated for 2 hr at room temperature with a 1:10 dilution of a supernatant of the monoclonal antibody AB11 (kindly pro- vided by D. L. King and F. C. Kafatos), which is directed against an epitope of Usp in the DNA-binding domain (Khoury Christianson *et al,* 1992). After three 15-min washes in TNT (TBS containing 0.1% Triton X-100), blots were incubated for 1 hr in peroxidase-conjugated anti-mouse IgG (Sigma) diluted 1:1000 with TBS. They were then washed in TNT three times for 15 min each and stained with DAB-H_2O_2 (160 μg/ml 3,3-diaminobenzidine, .0001% H_2O_2 in 1 x TBS).

The Usp protein used as a positive control (kindly pro- vided by Russ Eldridge and Lois K. Miller) was produced with a baculovirus expression vector under polyhedron promoter control according to previously described procedures (O'Reilly *et al.,* 1992).

Intmunohistochemistry. Whole larval tissues were fixed in 4% paraformaldehyde and stained by horseradish peroxidase methods (MacDonald and Struhl, 1986). The fixed tissues were rinsed in 1× PBS and then washed twice for 30 min each in PBN (1× PBS, 1% bovine serum albumin, 0.5% NP-40). The tissues then were incubated for 2 hr in the AB-11 monoclonal antib0dy, diluted 1:10 with PBN. After three 30-min washes in PBS, the tissues were incubated in horse anti-mouse IgG biotinylated antibody (1:500, Vector Laboratories) for 1 hr. After re- peated rinsing in PBS, the tissues were incubated in Vectastain ABC reagent (Vector Laboratories) for 1 hr. The conjugated peroxidase activity was then revealed with a 0.05% 3-3'-diaminobenzidine (Sigma), 0.025% H_2O_2 solution. The staining reaction was stopped by several washes in PBS. The tissues were then mounted in 50% glycerol in PBS and immediately examined under the microscope.

Amplification and sequencing of mutant alleles. Genomic DNA served as a template for PCR since preliminary reactions with primers that flank the only known open reading frame in the *usp* locus indicated that this portion of the gene contains no introns. The first primer, just 5' to the open reading frame (+174-195; Henrich *et al,* 1990) contained an *Eco*RI overhang site (5'-GGGAATTCCCCAGCACCACATCACAAGCCC-3') and the complementary primer (+1723-1741; Henrich *et al.,* 1990) contained an overhanging *Bam*HI sequence (5'-TTTGGATCCGCGCCTTTAGAGTCGGGACC-3') to facilitate cloning and verification. Genomic DNA was extracted by the methods of Ballinger and Benzer (1989) from females carrying a duplication ($Dp(1:3)w^{vco}$) which includes a usp^{+} allele. These females were also homozygous on the X chromosome for one of two lethal mutations (Perrimon *et al.,* 1985), usp^{s} (formerly usp^{VE653}; Lindsley and Zimm, 1992) or usp^{4} (formerly usp^{VE849}). For these genotypes, therefore, two-thirds of the genomic templates were mutant. At least two independent PCR reactions (AmpliTaq; Perkin-Elmer) were carried out for each genotype using standard procedures and conditions (Saiki, 1990). An amplification product of predicted length was recovered and subsequently subcloned by sticky/blunt end ligation to the multiple cloning site of a Bluescript plasmid vector (Stratagene) pre- digested with *Eco*RI and *Sma*I. At least four insert-containing clones were recovered by X-Gal color selection from each of the PCR reactions. All recovered clones were then subjected to standard dideoxynucleotide sequencing (Sequenase; U.S. Biochemical) with primers derived from sequences within the wild-type *usp* cDNA sequence (Henrich *et al.,* 1990) and the reaction products separated on a 6% polyacrylainide gel by standard methods. Candidate mutational sites were verified by identifying one or more clones from each of the PCR reactions that involved the same nucleotide substitution. Except for those noted here, no instances of a mutation at the same nucleotide were observed in more than one clone.

In vitro translation. The plasmids carrying mutant or wild-type PCR products that specified either wild-type or mutant Usp proteins were selected for further analysis. Another plasmid (pCAl-EcR) was also constructed by cloning the *Bam*HI (+621)/*Hind*III (+3961) fragment of EcR-B1 eDNA into Blueseript plasmid vector (Koelle *et* at, 1991). *In vitro* transcription with T3 or T7 polymerase was carried out according to manufacturer's protocols (Stratagene). For *in vitro* translation, 0.5, μg of transcript was mixed with 35 μl of rabbit reticulocyte lysate (Promega) in a total reaction volume of 50 μl. Each transcript was also added to a second lysate reaction containing [^{35}S]methionine. The mixture was separated electrophoretically after incubation and the relative abundance of translated Usp and EcR products estimated by autoradiography. This measure confirmed the presence of translated protein and revealed that the mutant Usp proteins were slightly more abundant than the wild-type Usp and EcR products. For both *usp* mutations, three plasmids encoding mutant proteins were translated and tested.

Gel shift analysis. From the lysate, 3-μl portions of EcR and Usp *in vitro* translated proteins were mixed and incubated on ice for 15 min in a total volume of 16 μl containing 25 m*M* Hepes (pH 7.0), 1 m*M* Tris (pH 7.5), 9% glycerol (v/v), 90 m*M* KCl, 1 m*M* EDTA, 0.9 m*M* DTT, 2 μg of poly(dI-dC), poly(dI-dC), and 5 pmole of m13 20-mer oligonucleotides (used as nonspecific single-stranded DNA binding competitors). The reaction was incubated for another 15 min following addition of 40 fmole of end-labeled hsp27 EcRE (Yao *et al.,* 1992). The probe was prepared by annealing the two single- stranded oligonueleotides, 5'-AGACAAGGGTTCAAT-GCACTTGTCCAA-3' and 5'-TTGGACAAGTGCATTGAACCCTTGTCT-3', by previously reported methods (Antoniewski *et al.,* 1993). Electrophoresis was per- formed as described (Buratowski *et al.,* 1989) and the

intensity of autoradiographic signals produced by the retarded complexes was quantified using a Molecular Dynamics Phosphorimager.

Genetic strains and crosses. For experiments involving the *usp*^{3} and *usp*^{4} mutations (referred to collectively as *usp*^{x}), stocks of the general genotype: *y usp*^{x} *w/y usp*^{x} *w/y*; *Dp*(*1;3*)*w*^{vco} *P*[*y*$^{+}$]/+ and FM7/*y usp*^{x} *w* were prepared by standard crossing procedures. The duplication, *Dp(1;3)w*^{vco}, carries a *usp*$^{+}$ allele. A transformant copy of *y*$^{+}$ (referred to as P[*y*$^{+}$]; Geyer and Corces, 1987) was linked to the duplication-bearing chromosome. It resides within five recombination units on the distal side of the duplication, which itself lies on 31, and served as a marker for the duplication-bearing chromosome in a *y*+ genetic background. The duplication was also detectable in these genotypes because it carries a variegating allele of 9hite (*w*^{cvo}). The *usp*^{2} (formerly *usp*^{KA21}; Perrimon *et al.,* 1985; Lindsley and Zimm, 1992) mutation could not be sustained in homozygous females and therefore was maintained in an *FM7/usp*^{2} stock. From this strain, *usp*^{2}*/Y; Dp*(*usp*+) males were obtained through appropriate crosses, as needed. Similarly, heterozygous or homozygous *usp* mutant flies carrying a transformant copy of *usp*$^{+}$ (referred to as *P*[*usp*$^{+}$] here; Oro *et al.,* 1990) were prepared by standard cr0ssing procedures. At eclosion, females and males of the genotypes designated under Results were crossed and transferred to 18° or 29°C. After several days, flies were transferred to new vials and eggs collected once a day. Offspring were counted and scored upon adult eclosion for the phenotypes described.

RESULTS

Levels of nsp Transcripts in Embryonic and Larval Stages

The appearance of *usp* transcript was analyzed at several times during larval development in 0rder to assess the gene's potential role in the hormonal regulation of molting and metamorphic events. On Northern blots prepared with total RNA a major signal was detected at 2.7 kb under high stringency conditions in all the developmental stages tested with a probe derived from a 2.2- kb cDNA (Henrich *et al.,* 1990; Fig la). Among the total RNA samples, a smaller 1.7-kb signal was also detected at all developmental times, but this appeared to be an artifact caused by rRNA obscuring the detection of signals in the interval between 2.7 and 1.7 kb.

The 2.7-kb transcript in total RNA was found throughout embryogenesis, but its abundance receded noticeably in the early first larval instar and later reached another minimum during the early third instar. Between the early and mid third instar the level of total *usp* naRNA increased and later reached a maximum in the early wandering period of the third larval instar, even though the amount of *rp49* transcript, used as a control for loading variation, remained relatively low throughout this period, The abundance of *usp* transcript declined slightly by the white prepupal stage and continued to recede through the early pupal stage. For developmental times when direct comparisons involving *usp* mRNA levels were possible, the changes observed here were consistent with those reported previously (Oro *et al.,* 1990; Andres *et, al.,* 1993). The decline of *rp49* control transcript found after the prepupal stage in this study has also been noted previously (Andres and Cher- bas, 1992). Northern blots prepared from the nonpolyadenylated RNA fraction of these t0tal RNA samples produced signals that were similar in intensity and pat- tern to those obtained from total RNA (data not shown).

This suggested that a significant proportion of the *usp* mRNA was not polyadenylated or possessed a relatively short polyadenine tail (<25 nucleotides), an observation further supported by densitometry analysis. During all stages except early embryogenesis, less than 15% of *usp* niRNA was polyadenylated, whereas 40 to 90% of *rp49* mRNA was recovered from the polyadenylated fraction in these same RNA samples (data not shown).

Only a small proportion of *usp* mRNA was polyadenylated whose changes in abundance might be obscured in a total RNA sample. Because of this concern and the possibility of signal interference caused by rRNA, a polyadenylated RNA fraction from each developmental stage was subjected to Northern analysis. The amount tested represents an approximately 25-fold concentration of the polyadenylated portion of the total RNA fractions previously shown.

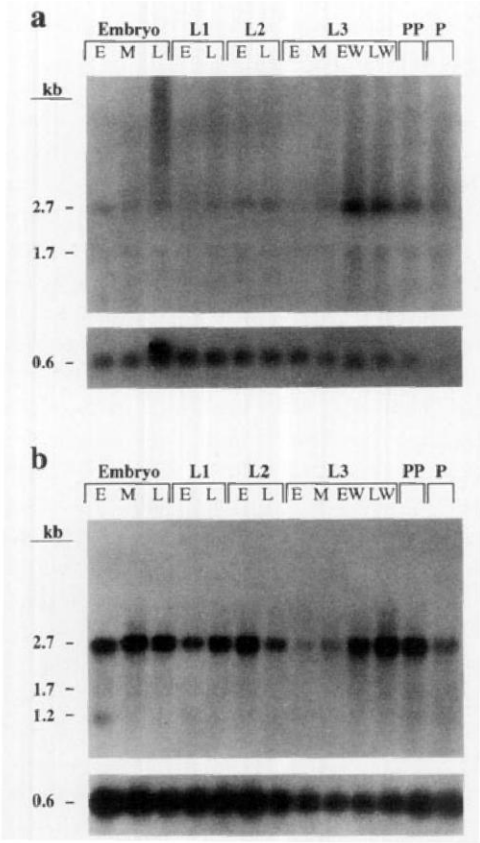

FIG. 1. (a) Levels of *usp* transcript in total RNA preparations from early embryogenesis through the early pupal stage. Each lane was loaded with 15 μg of total RNA. Boldfaced lettering indicates stage of life cycle: L1, first larval instar; L2, second larval instar; L3, third larval instar; PP, prepupal stage; P, early pupal stage. Letters within each stage designate early (E), middle (M), late (L), early wandering (EW), and late wandering (LW). Lower band indicates *rp49* RNA control. (b) Levels of *usp* and *rp49* transcript in polyadenylated RNA fractions derived from the total RNA samples are displayed in (a). Each lane contains 2 μg of polyadenylated RNA and represents about a 25-fold concentration of the polyadenylated RNA present in (a).

The developmental pattern of the polyadenylated RNA fraction was similar but not identical to the pat- tern seen with total RNA (Fig. lb). Levels of the polyadenylated 2.7-kb *usp* transcript reached a slight peak during mid embryogenesis, although it was easily detected throughout the embryonic stage. Another peak of the 2.7-kb transcript occurred during the early second larval instar which was not readily apparent in the total RNA fraction. The abundance of *usp* polyadenylated mRNA then receded gradually to relatively low levels in the early third larval instar. Whereas *rp49* transcript levels remained about the same throughout the third larval instar, the amount of *usp* mRNA increased during the mid third larval instar and reached its highest level in the late wandering third larval instar, several hours after the peak seen in total RNA fractions. The amount of transcript remained high through the white prepupal stage and then declined noticeably by the early pupal stage. Additionally, a 1.2-kb transcript not detected in the total RNA fraction was found in early embryogenesis, but it was considerably less abundant than the *larger* 2.7-kb transcript and not seen in any other embryonic, larval, or pupal stages.

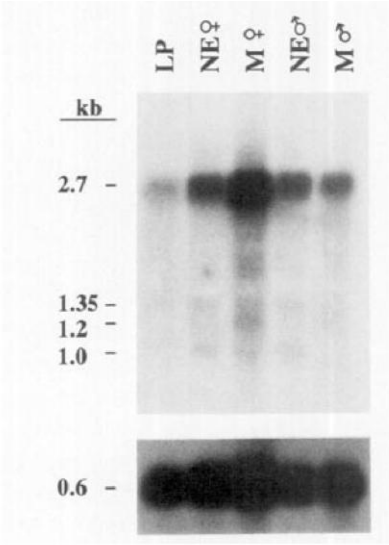

FIG. 2. Levels of *usp* transcript in polyadenylated RNA preparations from adult flies. Letters designate stages: LP, late pupal; NE, newly eclosed females and males (0–10 hr); M, mature females and males (92–100 hr). Each lane is loaded with 2 μg of polyadenylated RNA.

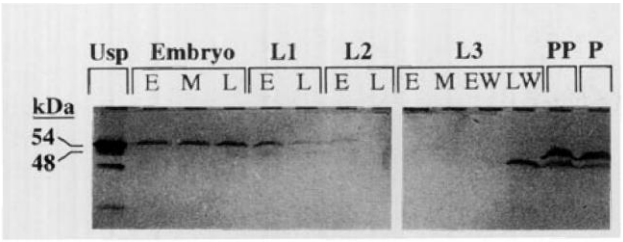

FIG. 3. Presence of Usp protein from the same developmental times depicted in Fig. 1 as detected by Western analysis with monoclonal antibody, AB11 (Khoury-Christianson *et al.*, 1992). Each lane contains 80 μg of protein. Space between L2 and L3 denotes two membranes from separate gels that were processed through all steps simultaneously. Lane designated as Usp indicates a baculovirus preparation used as a positive control.

Levels of usp Transcript in Adult Stages

In the late pupal stage, a 2.7-kb transcript was readily seen in polyadenylated RNA samples (Fig. 2). The abundance of the 2.7-kb *usp* mRNA had increased substantially by adult eclosion in both sexes, and the highest observed level among adult stages was found in 4-day- old adult females. A 1.2-kb transcript was also n0ted in mature females that presumably corresponds to the 1.2- kb transcript found in early embryos. Signals were also detected at 1.35 and 1.0 kb in all adult stages except mature males, although the larger size may be an artifact caused by rRNA interference. The existence of two minor *usp* transcripts in adult female ovaries has been noted previously (Shea *et al.,* 1990). A 5.0-kb signal was also found in mature males, but the possibility that this was contaminating DNA in the RNA sample was not completely ruled out.

Levels of Usp Protein

Developmentally staged animals were also tested for the presence of Usp protein (Fig. 3). The monoclonal antibody used, AB11, recognized two bands on a developmental Western blot (Khoury Christianson *et al.,* 1992). The larger 54-kDa band corresponds to the predicted size of lisp based on its deduced amino acid sequence. A smaller 48-kDa signal was also detected b0th in *Drosophila* protein extracts and in a bacuIovirus-driven expression system (Fig. 3). The 54-kDa protein was the most common form during embryogenesis and the early larval instars, whereas the 48-kDa form predominated in the wandering phase of the third larval instar. Both proteins were detected in the white prepupal and early pupal stages.

The detection of Usp roughly correlated with the presence of *usp* transcript and both were detected through- out embryogenesis. However, while polyadenylated *usp* transcript levels peaked in the early second larval in- star, levels of the protein declined progressively through the first and early second larval instar. The disparity between protein and transcript levels was greatest in the second larval instar, when the level of Usp declined below levels of detection, whereas *usp* mRNA was readily observed. Usp was not detected at all from the late second larval instar through the mid third larval instar, even though *usp* transcript was detected at varying levels throughout this period. The protein did not reappear at detectable levels until the early wandering stage and increased substantially during the late wan- dering third larval instar. These high levels were sustained through the early pupal period, 144 hr after egg laying. The late third larval instar RNA sample that was loaded onto the Northern blot (Fig. 1b) was derived from about 2.5 times as many larvae as were used to produce the equivalent protein sample (Fig. 3), although both samples were collected from the same culture. Assuming that this ratio is consistent for all samples, Usp protein may indeed be present during the late second and early third larval instars, but only at levels below the limit of detection on Western blots.

Expression of Usp in Larval and Imaginal Tissues

The abundance of lisp protein during late larval development is consistent with expectations about its role in mediating ecdysteroid-dependent expression during the onset of metamorphosis (Richards, 1992). In the wing discs, the presence of Usp in the nuclei of both epithelial cells and the peripodial membrane during the late third larval instar was readily apparent (Fig. 4a). The intensity of immunostaining was greatest among cell nuclei in the folds of these discs. Usp was also easily detected in the nuclei of both salivary gland and larval fat body cells of wandering third instar larvae (Fig. 4b). The fat body, like the salivary gland, responds transcriptionally to ecdysteroids (Deutsch *et al.*, 1989). The Usp protein was also found in the nuclei of all cell types that comprise the larval ring gland, the central nervous system, and the leg and eye-antennal discs during the wandering peri0d of the third larval instar (data not shown).

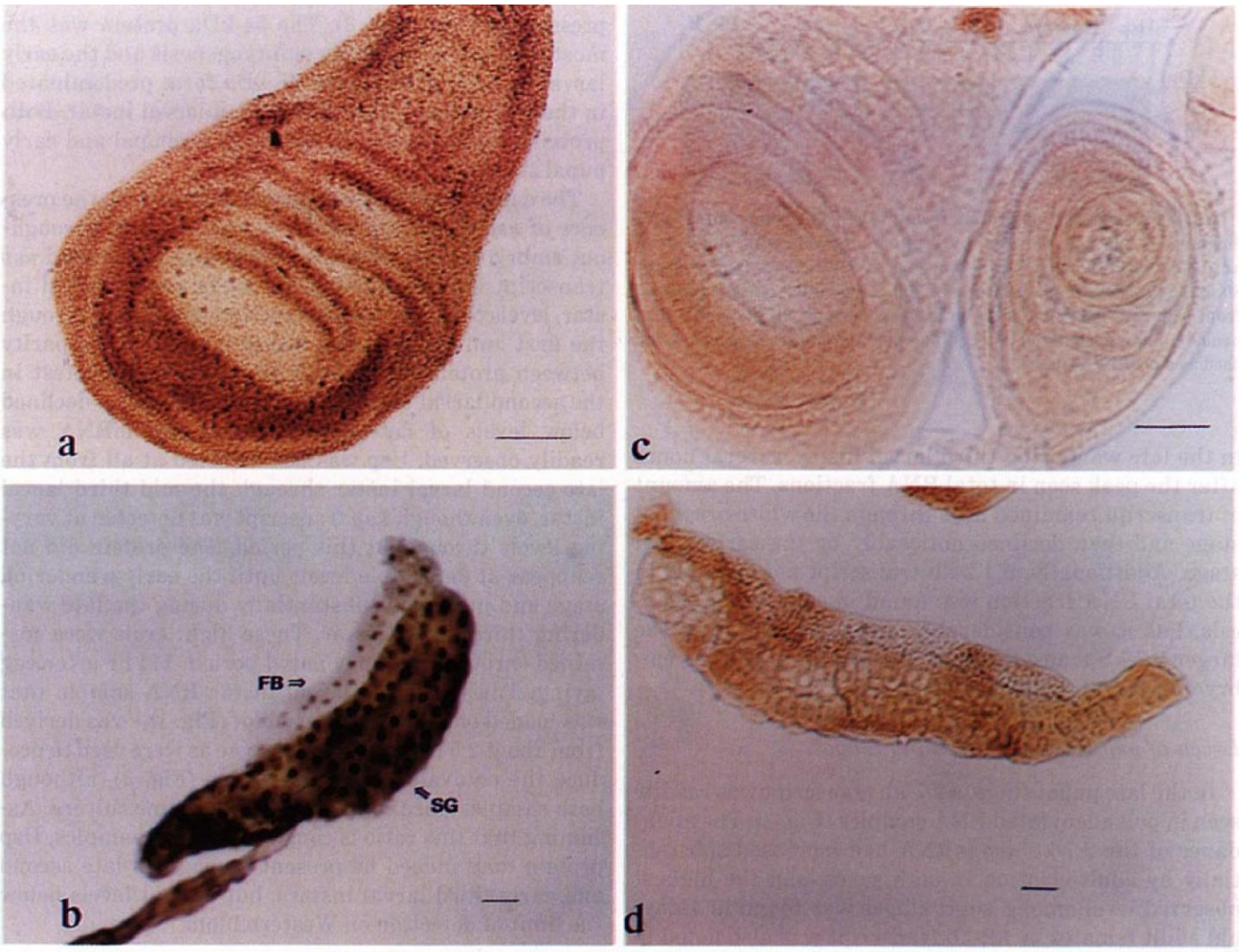

FIG. 4. Detection of Usp by immunostaining in individual tissues during late third larval instar as detected by AB11 monoclonal antibody. Marker equals 100 μm (a) Mesothoracic disc from late wandering third larval instar. (b) Salivary gland (SG) and fat body (FB) from late wandering phase of late third larval instar. (c) Negative control from wandering phase for mesothoracic disc. A leg disc is also shown. (d) Negative control for SG and FB from late wandering phase. In the negative controls, tissues were processed as described except that the mouse IgG (20 μg/ml of control medium; DMEM + 10% fetal calf serum) was used instead of AB11.

A Developmental Phenotype Associated with usp Mutations

As noted earlier, *usp* function may not be required for wing disc development during metamorphosis, even though Usp is readily detectable there in late third in- star larvae. However, the *usp* locus fulfills a vital function associated with wing disc development prior to the larval stage (Oro *et al.*, 1992).

A thoracic abnormality was found among adult females homozygous for either of two *usp* lethal mutations (*usp*^{3} and *asp*^{4} Lindsley and Zimm, 1992) on the X-chromosome who also carried a wild-type *usp* allele contained in the duplication, *Dp(1;3)w*^{vco} (Table 1). In these aneuploids, the relative dosage of normal and mutant lisp activity is roughly intermediate between the equal amounts found in heterozygotes and the absence of normal Usp activity in homozygous recessive larvae. The mildest mutant phenotype resulted in a slight separation of the microchaetes and macrochaetes along the dorsal midline of the thorax. A more severe phenotype resulted in a cleft which may have arisen either from the improper fusion of the discs along the dorsal midline of the notum or a mutant effect on cells lying along the midline of the mesothoracic disc. In the most severe phenotype, a cleft extended through both the notum and scutellurn of the dorsal thorax, although the epidermis was continuous in these flies, implying that some aspects of disc fusion occurred normally (Fig 5). Typically, these flies also possessed bent and misshapen sensory bristles. Many flies of this genotype also developed severely gnarled legs (data not shown), which are derived during early embryogenesis from the same ancestor cells as the wing discs (Cohen *et al.*, 1993).

TABLE 1
PERCENTAGE OF INDIVIDUALS DISPLAYING CLEFT THORAX PHENOTYPE AT 18°C AMONG PROGENY OF FEMALES HETEROZYGOUS FOR *usp* MUTATIONS

Cross (♀♀ × ♂♂)	Progeny genotypes		All other genotypes
	usp$^+$/*usp*$^+$; *Dp(usp*$^+$*)*/++	*usp*$^+$/Y; *Dp(usp*$^+$*)*/++	
(a)			
FM7/*usp*^{2} (1:1) × *usp*^{2}/Y; *Dp(usp*$^+$*)*/++	None recovered	6.2% (16)	0% (70)
FM7/*usp*^{3} (1:1) × *usp*^{3}/Y; *Dp(usp*$^+$*)*/++	11.1% (27)	0 (21)	0 (96)
FM7/*usp*^{4} (1:1) × *usp*^{4}/Y; *Dp(usp*$^+$*)*/++	37.5 (16)	0 (13)	0 (62)
(b)			
FM7/*usp*^{4} (1:1) × *usp*^{4}/Y; *Dp(usp*$^+$*)*/++	16.7% (18)	7.4% (27)	0% (97)
FM7/*usp*^{4}; *Dp(usp*$^+$*)*/++ (2:1) × *usp*^{4}/Y; *Dp(usp*$^+$*)*/++	7.1 (14)	3.6 (28)	0 (72)

Note. Calculations are based on surviving adult progeny of crosses between individuals carrying specified combinations of *usp* mutations and the duplication, *Dp(1;3)w*^{vco}, which carries a *usp*$^+$ allele. *Dp(usp*$^+$*)* designates *P{y*$^+$*}*, *Dp(1;3)w*^{vco}. X chromosomes bearing *usp*^{3} and *usp*^{4} alleles were marked with *yellow* (*y*) and *white* (*w*). Number of progeny for each genotypic class is given in parentheses next to percentage. ++ Designates a wild-type chromosome 3. Ratio of *usp*$^+$:*usp*$^-$ in females is indicated in parentheses next to each cross.

The duplication rescued males hemizygous for *usp*^{2}, which is probably the most severe allele because it is a nonsense mutation (Oro *et al.*, 1990). By contrast, the single wild-type *usp*$^+$ allele in the duplication consistently failed to rescue females homozygous for *usp* even though *usp*$^+$ */usp*^{2} *heterozygotes* survived normally (Table la). Based on the assumption that *usp*^{2} is a severe loss of function mutation, the failure to rescue homozygous *usp*^{2} females leads to the hypothesis that the wild-type *usp*$^+$ allele in *Dp(1;3)w*^{vco} is expressed at a lower level than the X-linked *usp*$^+$ allele in heterozygotes, perhaps as a consequence of position effect variegation. In fact, the duplication is inserted into a heterochromatic region of 3L, carries a variegating allele of the *white* locus (*w*^{vco}; Fauvarque and Dura, 1993) and is dosage compensated properly in males (Lucchesi *et al.*, 1974). The latter feature may explain the duplication's ability to rescue hemizygous *usp*^{2}*/Y; Dp(1;3)wv*^{coe} males. Duplication-bearing females carrying *usp*^{2} along with either *usp*^{3} or *usp*^{4} survived at a frequency predicted from the survival rates of various sibling classes. However, some of these heteroallelic mutant *usp* females developed the cleft thorax phenotype (Table 1). Siblings from the cross who carried *usp*$^+$ on the X chromosome always developed normally.

When the mother carried two copies of usp^{+} (one copy on the duplication) and a single copy of usp^{4}, the frequency of the phenotype among usp^{4}/usp^{4}; $Dp(1;3)w^{vco}$ females was lower than when the mother was heterozygous for usp^{4} (Table 113), suggesting that maternal *usp* genotype affected the frequency of cleft thorax among progeny. This possibility was tested by observing the frequency of cleft thorax among the progeny of usp^{4}/usp^{6}; $Dp(1;3)w^{vco}$ mothers crossed to usp^{4}/Y; $Dp(1;3)w^{vco}$ or usp^{2}/Y: $Dp(1;3)w^{vco}$ males (Table 2a). From these mothers, over 40% of the duplication-bearing adult female progeny who were homozygous for usp^{4} or heteroallelic for usp^{4} and usp^{2} developed this mutant phenotype, confirming that an increase in the relative dosage of mutant alleles in the mother increased the frequency of cleft thorax among progeny. Duplication-bearing females homozygous for usp^{3} or heteroallelic for usp^{3} and usp^{2} also underwent defective disc development, but the frequency for both groups was lower than for usp^{4} (Table 2a). Maternal impairment of *asp* function alone infrequently led to the phenotype among progeny. Less than 2% of *asp* heterozygous females derived from usp^{x}/usp^{x}; $Dp(1;3)w^{vco}$ mothers who had inherited usp^{4} paternally developed a cleft thorax (Table 2b). In the two sustainable $usp^{x}/usp^{x}/Y$; $Dp(1;3)w^{vco}$ strains, a noticeable fraction of individuals died during early stages of metamorphosis inside the pupal case.

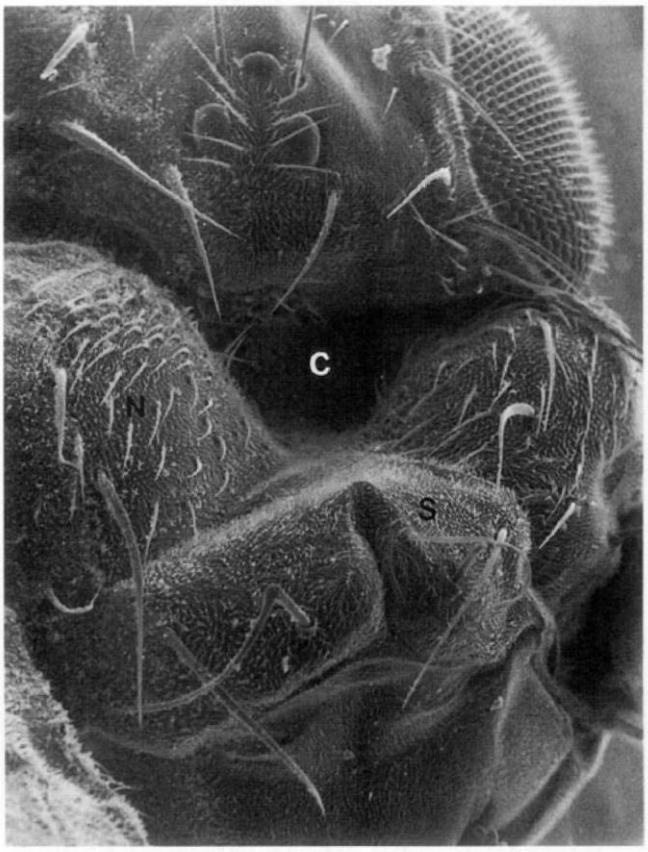

FIG. 5. Scanning electron micrograph of thoracic defect (cleft thorax) observed in usp^{4}/usp^{4}; $Dp(1;3)w^{vco}$ female flies and usp^{4}/Y; $Dp(1;3)w^{vco}$ male flies. View of dorsal thorax displays the failure of mesothoracic discs to fuse along dorsal midline resulting in cleft (C) through the notum (N) and scutellum (S).

Aside from the nature of the duplication itself, several lines of evidence further suggest that this phenotype arises from position effect variegation involving the wild-type asp^{+} allele within the duplication, First, usp^{4} homozygotic females carrying a transformed usp^{+} allele (Oro *et al.*, 1990) produced a smaller proportion of progeny who developed the cleft thorax trait than similar females carrying the duplication, suggesting that the usp^{+} allele in the latter is not as active as the transformed *usp* allele (Table 3). Second, the phenotype is more prevalent in females than males, consistent with the suppressive effects of the Y chromosome on position effect variegation (Table 4; Henikoff, 1990). Attempts to produce XO males or XXY females with chromosomes carrying a *usp* mutation failed, however, preventing a more direct test of this hypothesis. Third, the phenotype is

more frequent and severe at 18° than 29°C, as is also typical for position effect traits (Table 4; Fauvarque and Dura, 1993).

Flies transferred from 18° to 29°C during the second or third larval in star develop cleft thorax as frequently as flies kept at 18°C throughout the life cycle (Table 4). Therefore, maintenance at 18°C only during the developmental period that precedes metamorphosis was sufficient to evoke the higher occurrence of adults with cleft thorax caused by low temperature.

The presumed position effect trait was associated exclusively with individuals carrying *Dp(1;3)w^{vco}* and one or two *usp* mutations on the X chromosome. The phenotype was never found in stocks carrying either the duplication alone or only *usp* mutant alleles. Repeated attempts to segregate a second genetic factor responsible for the trait in either a wild-type or *usp* mutant background failed. Preliminary experiments also showed that flies are never homozygous for the duplication chromosome.

TABLE 2
PERCENTAGE OF INDIVIDUALS DISPLAYING CLEFT THORAX PHENOTYPE AT 18°C AMONG PROGENY OF *usp$^+$/usp$^+$; Dp(1;3)w^{vco}* FEMALES

Cross (♀♀ × ♂♂)	Progeny genotypes: *usp$^+$/usp$^+$; Dp(usp$^+$)/++*	Progeny genotypes: *usp$^+$/Y; Dp(usp$^+$)/++*
(a)		
usp^4/usp^4; Dp(usp$^+$)/++ × *usp^4/Y; Dp(usp$^+$)/++*	41.4 (58)	23.3 (60)
usp^3/usp^3; Dp(usp$^+$)/++ × *usp^3/Y; Dp(usp$^+$)/++*	25.9 (54)	14.8 (54)
usp^4/usp^4; Dp(usp$^+$)/++ × *usp^3/Y; Dp(usp$^+$)/++*	46.5 (101)	15.4 (78)
usp^3/usp^3; Dp(usp$^+$)/++ × *usp^3/Y; Dp(usp$^+$)/++*	16.7 (72)	0 (69)
(b)		
usp^4/usp^4; Dp(usp$^+$) × *usp$^+$/Y; Dp(usp$^+$)*	2.0 (101)*	20.8 (53)

* *usp^4/usp$^+$* and *usp^4/usp$^+$; Dp(usp$^+$)* female

Note. *Dp(usp$^+$)* designates P[*y$^+$*], *Dp(1;3)w^{vco}*. All X chromosomes bearing *usp$^+$*, *usp^3*, and *usp^4* alleles were marked with *yellow* (*y*) and *white* (*w*). ++ Denotes wild-type chromosome 3. Numbers in parentheses indicate number of individuals in each genotype class.

TABLE 3
PERCENTAGE OF INDIVIDUALS AT 18°C DISPLAYING CLEFT THORAX PHENOTYPE AMONG PROGENY OF A MASS MATING CROSS BETWEEN PARENTS CARRYING A TRANSFORMED *usp$^+$* ALLELE (DESIGNATED P[*usp$^+$*]; ORO *et al.*, 1990) AND *Dp(1;3)w^{vco}* (DESIGNATED *Dp(usp$^+$)*)

	I		II	
Genotype	*N*	Percentage	*N*	Percentage
usp^4/usp^4; Dp(usp$^+$)/Sb	27	14.8%	25	24.0%
*usp^4/usp^4; Dp(usp$^+$)/*P[*usp$^+$*]	20	0	13	0
usp^4/usp^4; P[*usp$^+$*]/+	10	0	21	0
usp^4/Y; Dp(usp$^+$)/Sb	29	3.6	15	0
*usp^4/Y; Dp(usp$^+$)/*P[*usp$^+$*]	28	0	30	3.3
usp^4/Y; P[*usp$^+$*]/+	10	0	19	0

Note. Roman numerals designate replications and *N* designates number of individuals in each genotypic class. The cross involves the following genotypes: *y usp^{4}w/y usp^{4}w;* P[*usp$^+$*]*/Sb* × *y usp^{4}w*/Y; *Dp(usp$^+$)*[*y$^+$*]/+.

Molecular Mapping of Mutations

In order to ascertain more precisely the function(s) potentially disrupted by the two EMS-induced mutations, the mutational site was characterized for each one. This was accomplished by employing primers which flank the open reading frame of *usp* to amplify genomic DNA from homozygous *usp* mutant female flies who also carried *Dp(1;3)w^{vco}*. For both *usp^3* and *usp^4*, the c0mplete sequence of several individual clones from multiple PCR reactions was compared. Both mutations involved amino acid substitutions of universally conserved arginine residues in the DBD. In each case, these were the only substitutions within the entire open reading frame that occurred repeatedly among clones derived from separate PCR reactions (Fig. 6a). The *usp^4* mutation is caused by a C to T nucleotide transition and converts an arginine (Arg130; Henrich *et al.,* 1990) to a cysteine residue in the linker region between the two zinc fingers (Fig, 6b) and lies within an a-helix implicated in DNA recognition (Freedman, 1992; Lee *et al.,* 1993). The *usp^3* mutation is caused by a *G* to A nucleotide transition that results in the substitution of a histidine for an arginine (Arg160; Henrich *et al.,* 1990) in another a-helix that resides in the second zinc finger (Fig. 6b; Lee et *al.*, 1993).

Interactions between Usp Mutant Proteins and EcR-B Protein

The functional ecdysteroid receptor is a heterodimer complex of the EcR and Usp proteins (Yao *et al.*, 1992, 1993; Thomas *et al.*, 1993). In order to assess the effects of mutant Usp^3 and Usp^4 proteins, translation products were tested for their ability to form complexes with the EcR-B1 isoform and interact with the hsp27 EcRE. When tested with radiolabeled hsp27 EcRE on gel shift assays, wild-type Usp protein and EcR-B1 produced a prominent retarded complex after electrophoresis un- der nondenaturing conditions (Fig. 7a). Neither protein alone formed a retarded complex (data not shown). By contrast, almost no retarded Usp^3/EcR-B1 complexes were observed, and the level of retarded Usp^4/EcR-B1 complexes was reduced by over 60% as indicated by autoradiographic densitometry (Fig. 7b). These results were repeated with each of three constructs encoding each mutant protein. The reduced affinity of these complexes for an EcRE is predictable since both mutations cause amino acid substitutions in portions of the DBD implicated in DNA recognition. It was predicted, however, that these mutant proteins would retain the ability to form dimers with EcR-B1. When equal amounts of EcR-B1, Usp^+, and Usp^3 proteins were incubated together with the hsp27 EcRE, the intensity of the retarded complex was reduced from 93% in a control group to 42%, strongly suggesting that Usp^3 protein retains completely normal dimerization function. Similarly, Usp^4 apparently competes normally with Usp^+ to form dimers with EcR-B1.

DISCUSSION

The profile of *usp* transcription and translation assembled here shows that while expression is not con- fined to developmental periods and cell types associated specifically with major ecdysteroid-induced events, it undergoes numerous and frequent changes during embryonic, larval, and adult development. In fact, the fluctuations observed in *usp* mRNA levels in other stages may partly reflect the heterogeneity of ecdysteroid-regulated transcription that is already well-established for the third larval instar (Karim and Thummel, 1992; Andres and Cherbas, 1992; Andres *et al.*, 1993; Huet *et al.*, 1993) and/or ecdysteroid-independent functions of the Usp gene product.

While *usp* transcript was evident thr0ughout embry- onic and larval development, its relative abundance in both total and polyadenylated RNA fractions was lowest during the early third larval instar and highest just prior to and through the white prepupal stage. The greatest rise in *usp* mRNA levels occurred between 94 and 106 hr after egg laying; Andres *et al.* (1998) have reported that this rise occurs primarily between 104 and 106 hr. The increase coincides with the greater abundance of Usp protein and the onset of ecdysteroid-induced events at metamorphosis. H0wever, it is currently unknown whether the accumulation of *usp* transcript and/or Usp protein in the late third larval instar is itself an ecdysteroid-induced response.

TABLE 4
EFFECT OF TEMPERATURE AND SEX ON FREQUENCY OF CLEFT THORAX PHENOTYPE

Rearing conditions	Progeny genotype	
	usp^4/usp^4; $Dp(usp^+)/++$	usp^4/Y; $Dp(usp^+)/++$
18°C	48.6% (37)	17.6% (57)
29°C	7.7% (28)	2.9% (34)
18°C, then 29°C	48.8% (90)	13.2% (98)

Note. Percentages are based on surviving progeny from a cross between usp^4/usp^4; $Dp(1;3)w^{vco}$ females and usp^4/Y; $Dp(1;3)w^{vco}$ males. $Dp(usp^+)$ designates P[y^+], $Dp(1;3)w^{vco}$. All X chromosomes were marked with *yellow* (*y*) and *white* (*w*). ++ Designates wild-type chromosome 3. Temperature transfer of fly cultures from 18° to 29°C was conducted 4–8 days after egg laying and before the wandering phase of the third larval instar. Number in parentheses indicates sample size.

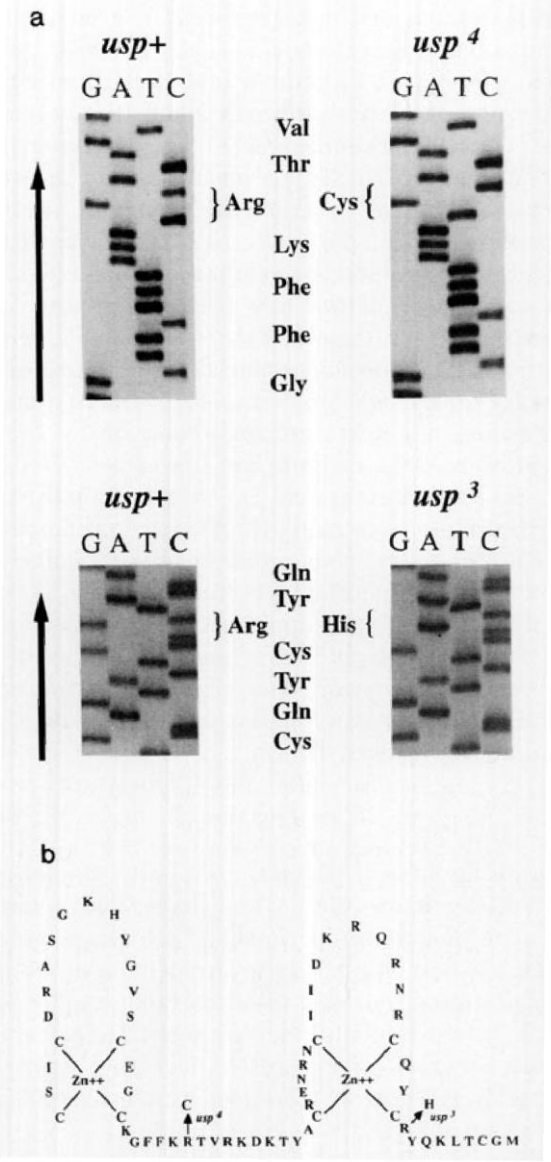

FIG. 6. Site of nucleotide substitutions of mutations in the *usp* locus. (a) Site of nucleotide substitutions in usp^4 and usp^3 verified by comparison of mutant and wild-type clones. (b) Schematic diagram of *usp* DNA-binding domain designating amino acid substitutions associated with the mutations.

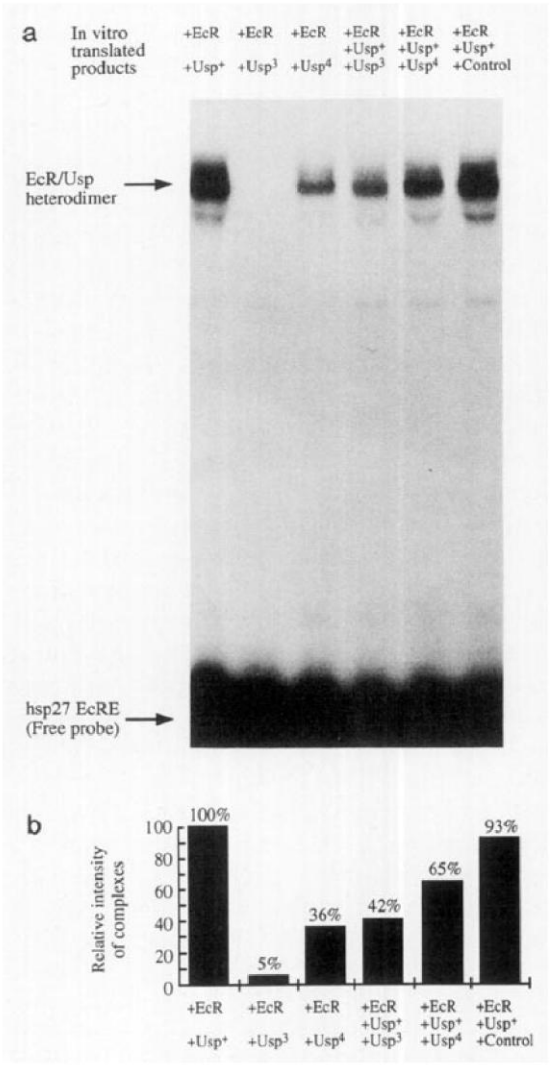

FIG. 7. Formation of EcR-B1/Usp/DNA complexes using Usp^+, Usp^3, and Usp^4 *in vitro* translated proteins. (a) Gel shifts using equal amounts of indicated *in vitro* translated proteins and radiolabeled hsp27 EcRE. Control in right lane refers to rabbit reticulocyte lysate containing no *in vitro* translated protein. (b) Relative densitometry of gel shift images shown in (a).

The greatest degree of transcriptional complexity appeared in adult stages, although the biological significance of the shorter *usp* transcripts reported here will require more detailed molecular analysis. The relative abundance of *usp* mRNA in adult females is consistent with previous indications that the *usp* gene plays an essential role in oogenesis and subsequent embryonic development (Perrimon *et al.*, 1985; Shea *et al.*, 1990; Oro *et al.*, 1992). It is uncertain whether the 1.2-kb adult female and early embryonic mRNA is a *usp* transcript that arises fr0m alternative splicing or an alternative promoter, although it is plausible that it is maternally sequestered into the egg. The signal may also represent a different gene, although the results of genomic Southern blots strongly argue that *usp* cDNA probes recognize a single gene (Oro *et al.*, 1990; Szekely and Henrich, unpublished). If the 1.2-kb signal indicates a *usp* transcript, it does not include the entire open reading frame of Usp, which is approximately 1.6 kb in length (Oro *et al.*, 1990; Henrich *et al.*, 1990). In fact, the AB11 antibody employed in this study recognizes smaller proteins in ovarian nuclear extracts that are more concentrated for Usp than whole body extracts (Khoury Christianson *et al.*, 1992). Interestingly, truncated versions of Usp recognize a regulatory region of the s15 chorion gene promoter and short *asp* transcripts have been reported in follicle cell preparations (Shea *et al.*, 1990), although it remains to be seen whether any of the short transcripts described here encode one or more truncated forms of Usp (Khoury Christianson *et al.*, 1992).

It is important to note that *usp* is transcribed in both sexes and that the expression profile changes with age, meaning the gene likely plays other roles not related to female reproduction. For example, Usp is present in male accessory organs (Kim and Gilbert, unpublished).

Indications of Usp Translational Regulation

A 48-kDa Usp protein was also detected repeatedly on whole body Western blots primarily during the late third larval instar and prepupal stage, whereas the 54-kDa form predominated in embryonic and early larval stage preparations. The smaller protein apparently lacks the most N-terminal portion of Usp, a domain associated with trans-activation (Khoury Christianson *et* at, 1992; Beato, 1989). Both *in vitro* translated forms arise from a single *usp* mRNA transcribed *in vitro,* suggesting the possibility that an alternative translational start site is involved in the synthesis of these forms which may be regulated developmentally (Chao and Gilbert, unpublished). The disparity which sometimes existed between transcript and protein levels, particularly in the early second larval instar, further suggests that Usp may undergo some form of translational regulation.

Developmental Basis for the Cleft Thorax Phenotype

Based on all available information, the cleft thorax phenotype arises from the cumulative effects of both maternal and early postzygotic underrepresentation of normal *usp* transcript caused by position effect variegation, leading to a deficit of normal Usp product. Presumably, variegated expression of the usp^{+} allele is adequate to rescue some developing flies, but does not completely overcome the deleterious effects of mutant Usp product in some disc anlage, resulting in abnormal wing disc development. The failure to observe this presumed variegating phenotype in endogenous usp^{+} transformants and heat shock-induced usp^{+} transformants probably stems from the relatively high levels of usp^{+} expression these constructs confer to all cells (Oro *et al.,* 1990, 1992).

The wing disc abnormalities reported here may involve the failure of an EcR interaction. More often than predicted from results described here, usp^{+}/usp^{4} ; EcR^{+}/EcR^{-} females *(*EcR^{-} refers either to a chromosomal deletion or a lethal mutation of the *EcR* locus kindly provided by M. Bender and D. S. Hogness) derived from usp^{4}/usp^{4}; $Dp(1;3)w^{vco}$ mothers developed a cleft thorax and hemizygous usp^{4}/Y; EcR^{+}/EcR^{-}; $Dp(1;3)w^{vco}$ males rarely survived to adulthood (Henrich, unpublished). Presumably, the proportion of functional Usp/EcR complexes was reduced below a critical threshold in the double heterozygotic females, thus leading to the abnormalities typically seen only in *usp* hemizygotes or homozygotes carrying the duplication. In males whose only source of normal Usp activity was derived from the variegating usp^{+} allele, the failure to form a critical number of normal EcR/Usp heteromers resulted in lethality. The visible adult abnormalities caused by allelic combinations of other *EcR* lethal mutations and the *usp* lethal mutations described here have been confined to wing and leg discs (Talbot *et al.,* 1993).

The early critical period associated with the cleft thorax phenotype and the presence of a 1.2-kb *usp* transcript that is confined to mature females and early embryos together suggest that important aspects of *usp* regulation remain to be identified at this stage. It cannot be surmised whether ecdysteroids mediate any aspect of this process, although strong alleles of the *ecdysoneless* gene locus, originally defined by an ecdysteroid-deficient conditional mutation, also fulfill a maternally derived vital function during embryogenesis (Hen- rich *et al.,* 1993).

The morphology of cleft thorax itself resembles the effects of a nonlethal mutation within the decapentaplegic complex (Segal and Gelbart, 1985) and also the effects of heat shock-induced expression of *Antennapedia* in the late third larval instar (Gibson and Gehring, 1988). The gnarled legs observed in the posterior legs of some "position effect flies" closely resembles the malformed phenotypes evoked by specific mutant alleles of *BR-C,* an early ecdysteroid-responsive gene (Kiss *et al.,* 1988). The phenotypic similarity could reflect a mutationally induced impairment of *BR-C* transcription in the leg disc or an aberrant interaction between the *BR- C* gene product and Usp.

The early critical period for the cleft thorax phenotype suggested by these experiments leaves unknown the functional significance of Usp's presence in the late third instar wing disc. It is possible that the cleft thorax phenotype could be evoked at metamorphosis by position effect variegation, but the ability to produce *usp* mutant clones in the wing disc that are normal in appearance and frequency suggest that this will not be the case (Oro *et* at, 1992). Ecdysteroid-induced genes activated by the EcR/Usp complex in the larval salivary gland are also transcriptionally induced in the wing imaginal disc (DiBello *et al.,* 1991; Boyd *et al.,* 1991; Huet *et al.,* 1993). Additionally, *usp* behaves as an organismal lethal mutation at metamorphosis, an observation confirmed both by previous studies which showed that conditional expression of usp^{+} during early larval lethal phases allowed survival only into the third larval instar and early pupal period (Oro *et al.,* 1992) and the pupal lethality observed among some flies with the cleft thorax genotype. Apparently, the variegating usp^{+} allele does not always express an adequate amount of product at metamorphosis to permit survival through this period.

Lethal Mutations of usp Are Not Equivalent

Both genetic and *in vitro* gel shift results indicate that the usp^{3} and wsp^{4} missense mutations encode products that retain partial function. The Usp^{3} protein heterodimerized normally with EcR, but this complex displayed very little recognition for hsp27 EcRE in *vitro.* Usp^{4}/EcR complexes retained partial ability to recognize the hsp27 EcRE *in vitro.* Paradoxically, usp^{4} caused the appearance of the cleft thorax *in vivo* more frequently than usp^{3}. The greater frequency of the cleft thorax phenotype caused by *usph* could stem from the ability of the Usp^{4}/EcR complexes to interfere with the recognition of EcREs by normal Usp/EcR complexes in aneuploid cells carrying both mutant and wild-type Usp proteins. This antirnorphic effect probably does not occur in cells containing Usp^{+} and Usp^{3} proteins, since Usp^{3}/EcR complexes show little affinity for EcREs in *vitro.* Of course, the severity and frequency of the phenotype also depend upon the extent to which the usp^{+} allele in $Dp(1;3)w^{vco}$ variegates.

This explanation rests largely on the assumption that mutant Usp proteins significantly disrupt transcription. When either of the conserved residues affected by usp^{3} and usp^{4} are mutated in other nuclear receptors, affinity for DNA response elements is reduced to varying de- grees (Hollenberg and Evans, 1988; Schena *et al.,* 1989; Sone *et al.,* 1990; Saijo *et al.,* 1991) and transcriptional activation by the mutated receptor is virtually eliminated. Neither mutation affects an ahelical structure adjacent to the carboxyl side of the DNA-binding domain which may be essential for RXR and Usp heteromerization (Lee *et al.,* 1993), nor do these mutations lie in portions of the cysteine-cysteine zinc finger associated with dimerization (Freedman, 1992), consistent with the results of the gel shift experiments reported here.

In summary, these studies reveal that the *usp* gene plays an important role maternally and during early development for eventual mesothoracic disc development, although the mutations responsible for this effect are not equivalent. The regulation of *usp* transcription during this period also may be more complex than anticipated. Changes in *usp* abundance and the appearance of short and/or alternative forms of both *usp* mRNA and Usp protein indicate that the gene and its product undergo changes that are important for hormonal processes during *Drosophila* development.

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